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Discrimination of *Rhizobium tropici* and *R. leguminosarum* strains by PCR-specific amplification of 16S–23S rDNA spacer region fragments and denaturing gradient gel electrophoresis (DGGE)

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V.M. DE OLIVEIRA, H.L.C. COUTINHO, B.W.S. SOBRAL, C.T. GUIMARÃES, J.D. VAN ELSAS AND G.P. MANFIO. 1999. With the aim of detecting *Rhizobium* species directly in the environment, specific PCR primers for *Rh. tropici* and *Rh. leguminosarum* were designed on the basis of sequence analysis of 16S–23S rDNA spacer regions of several *Rh. tropici*, *Rh. leguminosarum* and *Agrobacterium rhizogenes* strains. Primer specificity was checked by comparison with available rDNA spacer sequences in databases, and by PCR using DNA from target and reference strains. Sequence polymorphisms of rDNA spacer fragments among strains of the same species were detected by denaturing gradient gel electrophoresis (DGGE). The specific PCR primers designed in this study could be applied to evaluate the diversity of *Rh. tropici* and *Rh. leguminosarum* by analysing the polymorphisms of 16S–23S spacer rDNA amplified from either whole-cell or soil-extracted DNA.

INTRODUCTION

Rhizobium is a genus of soil bacteria belonging to the family Rhizobiaceae, whose members are able to establish symbiotic relationships with a range of legume plants of agricultural and environmental importance, a process that results in biological nitrogen fixation (BNF) (Hirsh 1992).

The inoculation of cultivated leguminous plants with selected rhizobial strains is recommended to maximize the contribution of BNF to the nitrogen status of the host plant. However, inoculation with commercially available strains may not improve crop yields in the presence of high numbers of indigenous rhizobia in the soil, which may be more competitive for nodulation or nitrogen fixation, and are often better adapted to the prevailing soil and climate conditions (Anyango *et al.* 1995). Therefore, the evaluation and monitoring of the indigenous rhizobial population diversity in soils is of great importance in estimating the need for inoculum, and for the screening of novel, highly effective inoculant strains.

The diversity of *Rhizobium* populations has usually been determined by phenotypic and/or genotypic characterization of strains isolated from legume root nodules (Martínez-Romero 1994). This approach may be prone to bias as the isolates obtained represent the bacterial genotypes which were successful in the competition for nodulation of the particular host plant. Recent reports have demonstrated individual plant selection effects (Handley *et al.* 1998).

Methods for extraction of soil DNA (Ogram *et al.* 1988; Steffan *et al.* 1988; Smalla *et al.* 1993) coupled with the use of molecular probes and/or PCR primers for the specific detection of *Rhizobium* (Laguerre *et al.* 1993a, b; Streit *et al.*, 1993; Pooyan *et al.* 1994; Tas *et al.* 1994) may offer an alternative approach to studying the composition of, and monitoring, natural rhizobial populations.

Sequences of 16S rDNA are known to be highly conserved among bacteria (Woese 1987) and, in spite of the fact that

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strain-specific regions are sometimes identified, analyses of the genetic variation within this gene are not always sufficient to differentiate between strains within a species. In contrast, sequence analysis of the spacer regions between the 16S and the 23S rDNA can be used to differentiate organisms at the intraspecific level, given the relatively higher sequence divergence of this region compared with rRNA genes (Honeycutt *et al.* 1995; Nour *et al.* 1995; Gürtler and Stanisich 1996).

Denaturing gradient gel electrophoresis (DGGE) allows the separation of DNA fragments with identical length but different nucleotide sequences. This technique, combined with PCR applied to 16S rRNA segments, has recently been introduced in the field of molecular microbial ecology and is used not only to 'profile' complex microbial communities (Muyzer *et al.* 1993; Heuer *et al.* 1997), but also to infer the phylogenetic affiliation of the community members (Muyzer *et al.* 1995). It is also used to test the purity of bacterial strains, to monitor the isolation of bacteria from environmental samples (Brinkhoff and Muyzer 1997), and to study the dynamics of specific populations according to environmental variations (Ferris *et al.* 1996; Teske *et al.* 1996).

In this study, the sequence of the spacer region between 16S and 23S rDNA was analysed to design PCR-specific primers able to differentiate *Rh. tropici* and *Rh. leguminosarum* strains from those of *Agrobacterium rhizogenes*, a phylogenetically closely related organism. This strategy was chosen because of the lack of variability of the 16S rRNA sequences between *Rh. leguminosarum*/*Rh. tropici* and *A. rhizogenes*, as observed on the phylogenetic trees and sequence alignments of the Ribosomal Database Project (Olsen *et al.* 1992). In addition, preliminary restriction enzyme polymorphism analyses of the 16S–23S intergenic spacers among *Rhizobium* and *Agrobacterium* species revealed the occurrence of polymorphic sites (data not shown).

MATERIALS AND METHODS

Strains, growth conditions and DNA extraction

Rhizobium leguminosarum bv. *trifolii* CCT 4179 (←IAM 12613) and CCT 4488 (=LMG 6119), *Rh. leguminosarum* bv. *viceae* CCT 5087^T (←IAM 12609^T), *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T (←IAM 12612^T), *Rh. tropici* CCT 4160^T (=LMG 9503^T) and CCT 4164 (=LMG 9517), *Rh. meliloti* CCT 4167^T (=LMG 6133^T) and CCT 4169 (=LMG 6130), *Rh. loti* CCT 4063^T (=LMG 6125^T) and CCT 4159 (=LMG 4284), *Agrobacterium tumefaciens* CCT 5075 (←IBSBF 304; =IAM 1524) and CCT 5265 (←LMG 196), *A. rhizogenes* CCT 4832^T (←IAM 13570^T) and CCT 4842 (←IBSBF 642; =IAM 13571), and *A. vitis* CCT 6069 (←IBSBF 915) and CCT 6072^T (←IBSBF 919^T; =ATCC 49767^T) were obtained from Coleção de Culturas Tropical (CCT), Fundação 'André Tosello', Campinas, SP, Brazil; ATCC (American Type Culture Collection); IAM (Institute of Applied Microbiology, Tokyo, Japan); IBSBF (Instituto Biológico, Seção de Bacteriologia Fitopatológica, Campinas, Brazil); and LMG (Laboratorium voor Microbiologie Universiteit, Gent, Belgium). *Rhizobium* strains were grown in YM broth (0·5 g KH_2PO_4, 0·2 g $MgSO_4.7H_2O$, 0·1 g NaCl, 0·5 g yeast extract, 10·0 g mannitol and 0·5% bromothymol blue litre^{-1} distilled water), and *Agrobacterium* strains in Nutrient Broth (Difco), for 48 h at 28 °C. Genomic DNA extractions from pure cultures were performed according to Pitcher *et al.* (1989).

PCR amplification

The 16S–23S intergenic spacers were amplified using primers pHr (Massol-Deya *et al.* 1995), located at position 1518–1541 relative to the *Escherichia coli* 16S sequence, and p23 Suni322anti (Honeycutt *et al.* 1995), at position 322 relative to the *E. coli* 23S sequence, generating fragments of approximately 1·5 kb. PCR was performed using 50 ng bacterial DNA in 50 μl reactions containing 2·0 U *Taq* polymerase (CENBIOT, RS, Brazil), 1 × *Taq* buffer, 2·0 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} dNTP mix and 0·4 μmol l^{-1} of each primer. The PCR amplifications were carried out by using an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 3 min at 72 °C, and a final extension at 72 °C for 5 min, in a Perkin Elmer 9600 thermal cycler (Norwalk, USA).

Cloning and sequencing

PCR-amplified 16S–23S rDNA intergenic spacers from *Rh. leguminosarum*, *Rh. tropici* and *A. rhizogenes* strains were cloned into pGEM-T vector (Promega) and partially sequenced in an automated system (ABI 373; Perkin Elmer, Foster City, CA, USA).

Primer design and specificity tests

Sequence data were aligned and analysed using the GDE software environment (Genetic Data Environment, V.2.2; gopher://megasun.bch.umontreal.ca: 70/11/GDE). Primer specificity was checked by comparison with available GenBank/EMBL/ DDBJ database sequences (Genbank database release 107.0 and updates up to July 98). The specificity of the primers was then confirmed in PCR reactions with reference strains under the conditions described above, except that the temperature profile consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 30 s at 60 °C and 45 s at 72 °C. The amplification products were visualized in 1·4% agarose gels, stained with ethidium bromide.

DGGE analysis

The DGGE technique was carried out using an INGENY apparatus (INGENY phorU-2, Vlissingen, The Netherlands) at 100 V and 60 °C for 16 h in 0·5 × TAE buffer (20 mmol l^{-1} Tris-acetate, 10 mmol l^{-1} sodium acetate, 0·5 mmol l^{-1} Na_2EDTA; pH 7·4). The denaturing gradient (urea and formamide) in 6% acrylamide gels (acrylamide/N,N′-methylenebisacrylamide, 37/1) ranged from 45 to 65% (100% denaturant corresponding to 7 mol l^{-1} urea and 40% (v/v) deionized formamide). The bands were visualized under u.v. light after staining with SYBR Green (Molecular Probes) diluted 1 : 4000 in 0·5 × TAE buffer for 1 h in the dark.

RESULTS AND DISCUSSION

The 16S–23S rDNA sequences of strains CCT 4179, CCT 5087^T, CCT 4168^T, CCT 4160^T, CCT 4164, CCT 4832^T and CCT 4842 were deposited in the Genome Sequence DataBase (National Center for Genome Resources, http://www.ncgr.org) and GenBank under the accession numbers AFO91791 and AFO91790, AFO91793 and AFO91792, AFO91789 and AFO91788, AFO91801 and AFO91800, AFO91799 and AFO91798, AFO91795 and AFO91794, and AFO91797 and AFO91796, corresponding to the initial 700 bases and the final 600 bases of the 16S–23S rDNA, respectively.

Three specific primers for *Rh. leguminosarum* and/or *Rh. tropici* were designed based on the alignment of the rDNA spacer sequences : rhizo2f, *Rh. tropici* and *Rh. leguminosarum* forward primer (5′-gAT ggC ACC AgT CAg gTg AC-3′), rhizo3r, *Rh. tropici* and *Rh. leguminosarum* reverse primer (5′-ggA AgA CTT gAA YTT CCg A-3′) and trop1f, *Rh. tropici* forward primer (5′-Cgg ACR TgS CCC gAT AT-3′; base degeneracy : R = AG, S = CG, Y = CT).

Specificity checks against sequences in nucleotide databases showed that the rhizo2f forward primer was homologous, with only two mismatches, to a non-coding sequence in the beginning of the 16S–23S spacer region of two strains of *A. vitis* (accession numbers U45329 and U28505). The rhizo3r reverse primer showed partial homology to the tRNA-Ile gene sequence within the 16S–23S spacer region of *Bradyrhizobium japonicum* (one mismatch; Z35330) and *A. vitis* (two mismatches; U45329 and U28505), and presented three or more mismatches to sequences from several other bacteria. No homologous sequences in the database were found for the trop1f forward primer.

In PCR reactions, the primer pair rhizo2f/rhizo3r was shown to be highly specific for reference strains of *Rh. leguminosarum* and *Rh. tropici*, whereas the primer pair trop1f/rhizo3r was shown to be specific for reference strains of *Rh. tropici* (Figs 1 and 2, respectively). Neither primer pair amplified rDNA spacer fragments of the closely related species *A. rhizogenes*. The PCR products, varying in size between 200 and 400 bp, showed intraspecific length polymorphism for *Rh. leguminosarum* strains CCT 4179, CCT 5087^T and CCT 4168^T (Fig. 1, lanes 2, 4 and 5) and *Rh. tropici* strains CCT 4160^T and CCT 4164 (Fig. 1, lanes 6 and 7; Fig. 2, lanes 5 and 6). However, *Rh. leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488 (Fig. 1, lanes 2 and 3), which belong to the same biovar, did not present length polymorphism.

Denaturing gradient gel electrophoresis (DGGE) was applied to analyse the sequence polymorphism of the 16S–23S rDNA spacer fragments, and to evaluate the ability of this technique to differentiate *Rhizobium* spp. at the strain

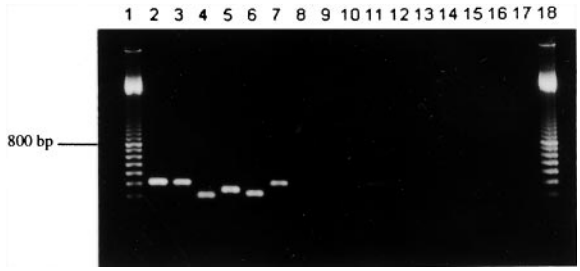

Fig. 1 PCR fragments amplified from pure cultures of reference strains using primer pair rhizo2f/rhizo3r. Lanes 1 and 18 : 100 bp marker (Pharmacia) ; lanes 2 and 3 : *Rhizobium leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488 ; lane 4 : *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T ; lane 5 : *Rh. leguminosarum* bv. *viceae* CCT 5087^T ; lanes 6 and 7 : *Rh. tropici* CCT 4160^T and CCT 4164 ; lanes 8 and 9 : *Agrobacterium rhizogenes* CCT 4832^T and CCT 4842 ; lanes 10 and 11 : *A. tumefaciens* CCT 5075 and CCT 5265 ; lanes 12 and 13 : *A. vitis* CCT 6069 and CCT 6072^T ; lanes 14 and 15 : *Rh. meliloti* CCT 4167^T and CCT 4169 ; lanes 16 and 17 : *Rh. loti* CCT 4063^T and CCT 4159

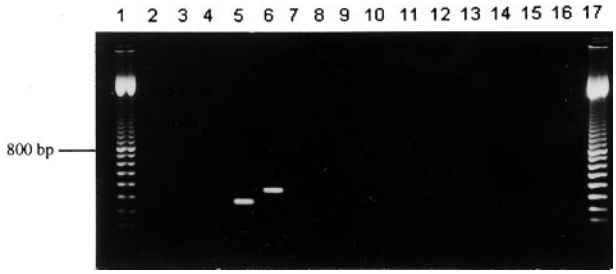

Fig. 2 PCR fragments amplified from pure cultures of reference strains using primer pair trop1f/rhizo3r. Lanes 1 and 17 : 100 bp marker (Pharmacia) ; lane 2 : *Rhizobium leguminosarum* bv. *trifolii* CCT 4179 ; lane 3 : *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T ; lane 4: *Rh. leguminosarum* bv. *viceae* CCT 5087^T ; lane 5 and 6 : *Rh. tropici* CCT 4160^T and CCT 4164 ; lanes 7 and 8 : *Agrobacterium rhizogenes* CCT 4832^T and CCT 4842 ; lanes 9 and 10 : *A. tumefaciens* CCT 5075 and CCT 5265 ; lanes 11 and 12 : *A. vitis* CCT 6069 and CCT 6072^T ; lanes 13 and 14 : *Rh. meliloti* CCT 4167^T and CCT 4169 ; lanes 15 and 16 : *Rh. loti* CCT 4063^T and CCT 4159

level. The pattern obtained (Fig. 3) confirmed the high resolution power of DGGE gels in separating DNA fragments of similar sizes but different sequence composition. The 16S–23S spacer fragments amplified from *Rh. leguminosarum* biovar *trifolii*, strains CCT 4179 and CCT 4488, which yielded same-sized PCR fragments, were clearly differentiated by DGGE (Fig. 3, lanes 2 and 3). Also, the PCR products of strains CCT 4168^T and CCT 5087^T, biovar *phaseoli* and *viceae*, respectively, which were barely differentiated in agarose gels, showed distinct melting behaviours in DGGE, resulting in a large migration distance between them (Fig. 3, lanes 4 and 5).

The DGGE technique has been largely used in studies of the diversity and dynamics of complex natural microbial communities (Muyzer *et al*. 1995 ; Ferris *et al*. 1996 ; Brinkhoff and Muyzer 1997) for the analysis of 16S rRNA gene fragments, which are separated on the basis of differences in sequence composition. However, given the limited fragment size that can be resolved by DGGE (Myers *et al*. 1985), the variability of 16S rDNA sequences may not be sufficient to discriminate phylogenetically closely related organisms at lower rank taxa.

In previous studies, it was demonstrated that DGGE analysis of 16S rDNA fragments (200 bp fragments amplified using bacterial universal primers) was not sufficient to discriminate among several *Rhizobium* species (Vallaeys *et al*. 1997). In contrast, 16S–23S rDNA fragments were shown to be highly variable in size and sequence (Laguerre *et al*. 1996), and thus could be of potential value for discriminating between phylogenetically related *Rhizobium* strains.

Data obtained from the current study corroborate the resolution power of 16S–23S rDNA sequences and DGGE in distinguishing phylogenetically closely related organisms, such as in the case of *Rh. leguminosarum*/*Rh. tropici* and *A. rhizogenes*, in contrast to the analysis of 16S rDNA alone. The results demonstrate that the rhizobial-specific primers designed in the present study, and DGGE, can also be used to differentiate rhizobia at the intraspecific level. The use of methods for extracting DNA directly from soil microbial communities, for specific 16S–23S rDNA spacer PCR amplification, and for DGGE, may provide a rapid, accurate and sensitive approach to characterizing and monitoring the diversity of *Rh. tropici* and *Rh. leguminosarum* in the environment.

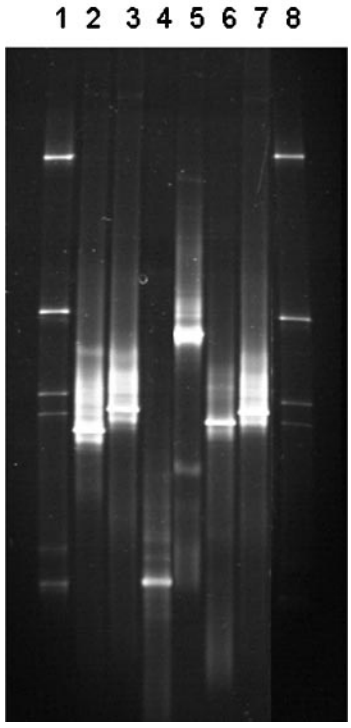

Fig. 3 DGGE pattern of the specific PCR fragments generated with the primer pair rhizo2f and rhizo3r/GC-clamp from DNA of reference strain pure cultures. Lanes 1 and 8 : DGGE marker (PCR fragments amplified using the primer pair U968/GC-clamp and L1401 from DNA of *Enterobacter cloacae* BE1 ; *Listeria innocua* ALM 105 ; *Rhizobium leguminosarum* bv. *trifolii* R62 ; *Arthrobacter* sp. and *Pseudomonas cepacia* P2, from top) ; lanes 2 and 3 : *Rh. leguminosarum* bv. *trifolii* CCT 4179 and CCT 4488, respectively ; lane 4 : *Rh. leguminosarum* bv. *phaseoli* CCT 4168^T ; lane 5 : *Rh. leguminosarum* bv. *viceae* CCT 5087^T ; lanes 6 and 7 : *Rh. tropici* CCT 4160^T and CCT 4164, respectively

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